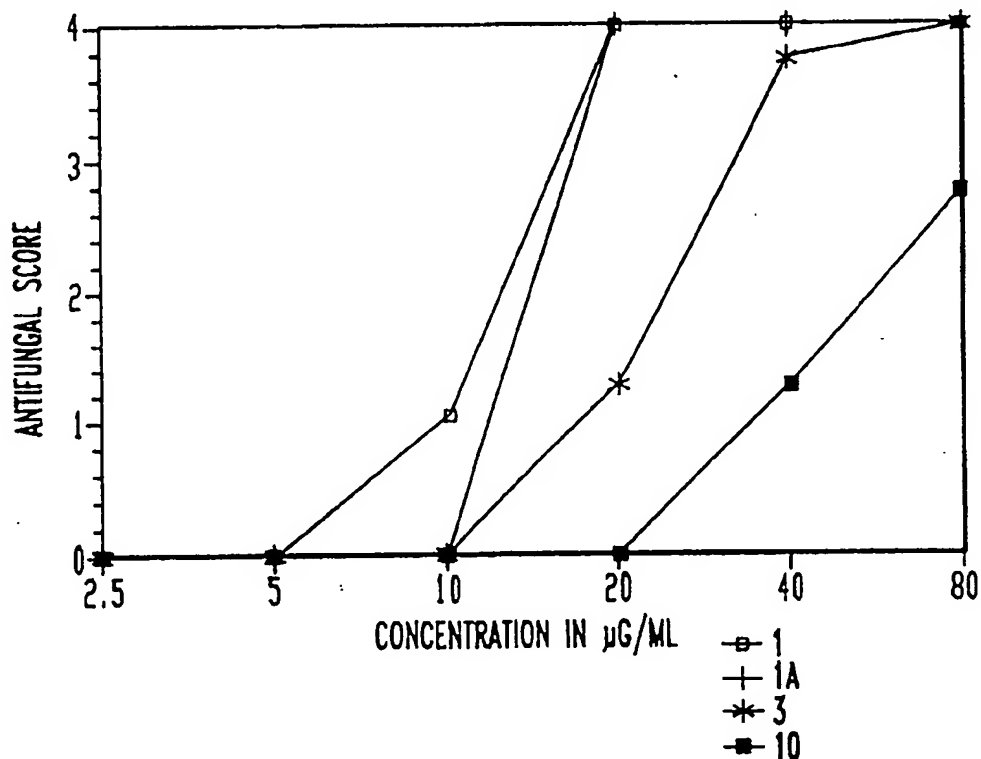




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(21) International Application Number: <b>PCT/US94/00382</b> (22) International Filing Date: <b>12 January 1994 (12.01.94)</b> (30) Priority Data: <b>08/003,885</b> <b>13 January 1993 (13.01.93)</b> <b>US</b> (71) Applicant: <b>PIONEER HI-BRED INTERNATIONAL, INC.</b> <b>[US/US]; 700 Capital Square, 400 Locust Street, Des</b> <b>Moines, IA 50309 (US).</b> (72) Inventors: <b>RAO, A., Gururaj; 4628 70th Place, Urbandale, IA</b> <b>50322 (US). BEACH, Larry, R.; 3939 Maquoketa Drive,</b> <b>Des Moines, IA 50311 (US).</b> (74) Agents: <b>ROTH, Michael, J. et al.; 700 Capital Square, 400</b> <b>Locust Street, Des Moines, IA 50309 (US).</b>		(81) Designated States: <b>AT, AU, BB, BG, BR, BY, CA, CH, CN,</b> <b>CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU,</b> <b>LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD,</b> <b>SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE,</b> <b>DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI</b> <b>patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE,</b> <b>SN, TD, TG).</b>  <b>Published</b> <i>Without international search report and to be republished          upon receipt of that report.</i>

(54) Title: HIGH LYSINE DERIVATIVES OF ALPHA-HORDOTHIONIN



(57) Abstract

Derivatives of  $\alpha$ -hordothionin made by position-specific substitution with lysine residues provide lysine enrichment while retaining the antifungal activity of the parent compound.

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## HIGH LYSINE DERIVATIVES OF ALPHA-HORDOTHIONIN

5 TECHNICAL FIELD

This invention relates to derivatives of  $\alpha$ -hordothionin which provide higher percentages of lysine while retaining the antifungal functionality of hordothionins.

10

BACKGROUND OF THE INVENTION

Disease resistance is an important objective of the genetic engineering of crop plants. Numerous fungi and bacteria are serious pests of common agricultural crops. One method of controlling diseases has been to apply antimicrobial organic or semiorganic chemicals to crops. This method has numerous, art-recognized problems. A more recent method of control of microorganism pests has been the use of biological control organisms which are typically natural competitors or inhibitors of the troublesome microorganisms. However, it is difficult to apply biological control organisms to large areas, and even more difficult to cause those living organisms to remain in the treated area for an extended period. Still more recently, techniques in recombinant DNA have provided the opportunity to insert into plant cells cloned genes which express antimicrobial compounds. This technology has given rise to additional concerns about eventual microbial resistance to well-known, naturally occurring antimicrobials, particularly in the face of heavy selection pressure, which may occur in some areas. Thus, a continuing effort is underway to express naturally occurring antimicrobial compounds in plant cells directly by translation of a single structural gene.

35 However, the use of such techniques gives rise to further problems. Crop plants have more important things to do than fight disease. They are sources of sugars, starches, proteins, oils, fibers, and other raw materials. Genetic

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engineers would also like to modify, and often to enhance, the production of those natural plant products. Unfortunately, plant cells can only produce large quantities of a few cellular components at a time. If they are producing high levels of storage proteins, it is difficult for them to also produce high levels of antifungal compounds. Thus, genetic engineers face a quandary in designing advanced plant systems with existing molecules for protein quality enhancement and disease resistance which require concurrent high-level expression of multiple genes.

### Brief Description of the Drawing Figures

Figure 1 is a graph of antimicrobial performance of various compounds discussed herein against *S. sclerotiorum*.

Figure 2 is a graph of antimicrobial performance of various compounds discussed herein against *A. flavus*.

Figure 3 is a graph of antimicrobial performance of various compounds discussed herein against *F. graminearum*.

Figure 4 is a graph of antimicrobial performance of various compounds discussed herein against *F. moniliforme*.

### DISCLOSURE OF THE INVENTION

It has now been determined that one class of compounds, the  $\alpha$ -hordothionins, can be modified to enhance their content of lysine while maintaining their antifungal activity. These hordothionin derivatives can be expressed to simultaneously enhance both resistance to fungal diseases and lysine content of the plant.

$\alpha$ -hordothionin is a 45-amino acid protein which has been well characterized. It can be isolated from seeds of barley (*Hordeum vulgare*) and even in its native form is especially rich in arginine and lysine residues, containing 5 residues (10%) of each. The amino acid sequence is as provided in SEQUENCE I.D. No. 1. It has powerful antifungal properties. Initial work to enhance the lysine content of this protein provided a high lysine derivative as indicated in SEQUENCE

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I.D. No. 2. However, it was impossible to predict the ultimate effect of this seemingly trivial substitution on the tertiary structure and folding of the protein, and subsequent bioassays determined that this derivative did not fold to a biologically active species in vitro. In addition, both tertiary structure and folding are critical to the stability and adequate expression of the protein in vivo, and both were absent in this compound. Therefore, further analysis and functional modeling of the wild-type compound was undertaken to determine whether substitutions could be made without disrupting biological activity. Although the crystal structure of crambin, a small protein of similar size and structure, has been reported, such crystal structures have not previously been available for hordothionin or even related compounds such as purothionin and viscotoxin. We undertook to develop such structural information.

Three-dimensional modeling of the protein led us to believe that the arginine residue at position 10 was critical to retention of the appropriate 3-dimensional structure and possible folding through hydrogen bond interactions with the C-terminal residue of the protein. A lysine substitution at that point with its shorter side chains could not hydrogen bond at the same time to both the serine residue at the 2 position and to the C-terminus while maintaining the backbone structure which we had predicted. The synthetic peptide having this substitution could not be made to fold correctly, which supported this analysis. Conservation of the arginine residue at position 10 provided a protein which folded correctly, had the sequence indicated in SEQUENCE I.D. No. 3, and exhibited antifungal activity in a bioassay. Comparison of the structure of hordothionin with that of the loosely related (48% homologous, 30% identical) protein crambin showed that thionin had a disulfide bond linking the cysteines at positions 12 and 29 which was not bridging the corresponding positions in crambin. Accordingly, replacement of the cysteine at position 12 of thionin with lysine and replacement of the cysteine at position 29 with threonine to produce a protein having the sequence indicated in SEQUENCE I.D. No. 4

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was found not to disrupt the 3-dimensional structure of the protein, as evidenced by an energy content which was determined to be indistinguishable from that of the native protein.

5 Further analysis of substitutions which would not alter the 3-dimensional structure of the molecule led to replacement of Asparagine-11, Glutamine-22 and Threonine-41 with lysine residues with virtually no steric hindrance. The resulting compound had the sequence indicated in SEQUENCE I.D. No. 5,  
10 containing 29% lysine residues. In addition, it was determined that by replacement of the serine residue at position 2 with aspartic acid, the arginine at position 10 could be replaced with lysine while permitting the needed hydrogen bonding with the C-terminus, providing a compound of  
15 the sequence indicated in SEQUENCE I.D. No. 6. It should be appreciated that that these substitutions would be effective and acceptable could not have been predicted by examination of the linear sequence of the native thionin protein.

Other combinations of these substitutions were also made,  
20 providing proteins having the sequences indicated in sequence i.d. no. 7 and sequence i.d. no. 8. accordingly, this invention provides proteins having the sequence of sequence i.d. no. 9 wherein the amino acid residues at one or more of positions 5, 10, 11, 12, 17, 19, 22, 30 and 41 are lysine, and  
25 the remainder of the residues at those positions are the residues at the corresponding positions in sequence i.d. no. 1, provided that the residue at position 30 is threonine when the residue at position 12 is lysine and cysteine otherwise, and the residue at position 2 is aspartic acid when the  
30 residue at position 10 is lysine and serine otherwise. although the native hordothionin is relatively lysine rich, a storage protein with 10% lysine residues (by number) cannot be expressed at high enough levels to obtain total protein lysine contents which are sufficient to obviate the need for lysine  
35 supplementation in poultry and swine feeds. these compounds are significantly more lysine enriched, and can be made to contain nearly thirty percent lysine residues. without such enhanced lysine contents, it is impossible to eliminate the

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need for lysine supplementation of feeds. this invention thus also provides an important method for enhancing the lysine content of a plant cell or a plant, comprising the step of causing one or more proteins according to this invention to be expressed in the cell or plant.

5 synthesis of the compounds was performed according to methods of peptide synthesis which are well known in the art and thus constitute no part of this invention. in vitro, we have synthesized the compounds on an applied biosystems model 10 431a peptide synthesizer using fastmoc<sup>tm</sup> chemistry involving hbtu [2-(1h-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, as published by rao et al., int. j. pep. prot. res. 40:508-515 (1992). peptides were cleaved following standard protocols and purified by reverse phase 15 chromatography using standard methods. the amino acid sequence of each peptide was confirmed by automated edman degradation on an applied biosystems 477a protein sequencer/120a pth analyzer. more preferably, however, the compounds of this invention are synthesized in vivo by 20 bacterial or plant cells which have been transformed by insertion of an expression cassette containing a synthetic gene which when transcribed and translated yields the desired compound. such empty expression cassettes, providing appropriate regulatory sequences for plant or bacterial 25 expression of the desired sequence, are also well-known, and the nucleotide sequence for the synthetic gene, either rna or dna, can readily be derived from the amino acid sequence for the protein using standard reference texts. Preferably, such synthetic genes will employ plant-preferred codons to enhance 30 expression of the desired protein.

### Industrial Applicability

The following description further exemplifies the 35 compositions of this invention and the methods of making and using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

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## Plants

The polypeptides employed in this invention can be effectively applied to plants afflicted with susceptible microorganisms by any convenient means, including spray, 5 creams, dust or other formulation common to the antimicrobial arts. The compound can also be incorporated systemically into the tissues of a treated plant so that in the course of infesting the plant the pathogens will be exposed to antimicrobial amounts of the compound of this invention. One 10 method of doing this is to incorporate the compound in a non-phytotoxic vehicle which is adapted for systemic administration to the susceptible plants. This method is commonly employed with fungicidal materials such as captan and is well within the purview of one of ordinary skill in the art 15 of plant fungicide formulation. However, since the genes which code for these compounds can be inserted into an appropriate expression cassette and introduced into cells of a susceptible plant species, an especially preferred embodiment of this method involves inserting into the genome of the plant 20 a DNA sequence coding for a compound of this invention in proper reading frame, together with transcription initiator and promoter sequences active in the plant. Transcription and translation of the dna sequence under control of the regulatory sequences causes expression of the protein sequence 25 at levels which provide an antimicrobial amount of the protein in the tissues of the plant which are normally infected by the pathogens.

the plant is preferably a plant susceptible to infection and damage by one or more of fusarium graminearum, fusarium 30 moniliforme, aspergillus flavus, alternaria longipes, sclerotinia sclerotiorum, and sclerotinia trifoliorum. these include corn (zea mays) and sorghum (sorghum bicolor). however, this is not to be construed as limiting, inasmuch as these two species are among the most difficult commercial 35 crops to reliably transform and regenerate, and these pathogens also infect certain other crops. thus the methods of this invention are readily applicable via conventional techniques to numerous plant species, if they are found to be



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susceptible to the plant pathogens listed hereinabove, including, without limitation, species from the genera allium, antirrhinum, arabidopsis, arachis, asparagus, atropa, avena, beta, brassica, browallia, capsicum, cicer, cicla, citrullus,  
5 citrus, cucumis, cucurbita, datura, daucus, digitalis, fagopyrum, fragaria, geranium, glycine, gossypium, helianthus, hordeum, hemerocallis, lactuca, lens, lolium, lotus, lycopersicon, majorana, manihot, medicago, nasturtium, nicotiana, oryza, pelargonium, persea, petunia, phaseolus,  
10 pisum, ranunculus, raphanus, ricinus, saccharum, secale, senecio, setaria, solanum, spinacia, trifolium, triticum, bromus, cichorium, hyoscyamus, linum, nemesia, panicum, onobrychis, pennisetum, salpiglossis, sinapis, trigonella, and vigna.

15 preferred plants that are to be transformed according to the methods of this invention are cereal crops, including maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean.

synthetic dna sequences can then be prepared which code  
20 for the appropriate sequence of amino acids, and this synthetic dna sequence can be inserted into an appropriate plant expression cassette.

likewise, numerous plant expression cassettes and vectors are well known in the art. by the term "expression cassette"  
25 is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. expression cassettes frequently and preferably contain an assortment of restriction sites suitable  
30 for cleavage and insertion of any desired structural gene. it is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. in addition, the plant expression cassette preferably includes a strong constitutive promoter sequence at one end to cause the  
35 gene to be transcribed at a high frequency, and a poly-a recognition sequence at the other end for proper processing and transport of the messenger rna. an example of such a preferred (empty) expression cassette into which the cdna of

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the present invention can be inserted is the pphi414 plasmid developed by beach et al. of pioneer hi-bred international, inc., johnston, ia, as disclosed in u.s. patent application no. 07/785,648, filed october 31, 1991. highly preferred  
5 plant expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

by the term "vector" herein is meant a dna sequence which is able to replicate and express a foreign gene in a host  
10 cell. typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance, which then serve  
15 as markers to identify and separate transformed cells. preferred markers/selection agents include kanamycin, chlorosulfuron, phosphonothricin, hygromycin and methotrexate. a cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector  
20 and is referred to as a "transformant."

a particularly preferred vector is a plasmid, by which is meant a circular double-stranded dna molecule which is not a part of the chromosomes of the cell.

as mentioned above, both genomic and cdna encoding the  
25 gene of interest may be used in this invention. the vector of interest may also be constructed partially from a cdna clone and partially from a genomic clone. when the gene of interest has been isolated, genetic constructs are made which contain the necessary regulatory sequences to provide for efficient  
30 expression of the gene in the host cell. according to this invention, the genetic construct will contain (a) a first genetic sequence coding for the protein or trait of interest and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. typically,  
35 the regulatory sequences will be selected from the group comprising of promoters and terminators. the regulatory sequences may be from autologous or heterologous sources.

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promoters that may be used in the genetic sequence include nos, ocs and camv promoters.

an efficient plant promoter that may be used is an overproducing plant promoter. overproducing plant promoters  
5 that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-biphosphate carboxylase from soybean (berry-lowie et al., j. molecular and app. gen., 1:483-498 (1982)), and the promoter of the  
10 cholorophyll a-b binding protein. these two promoters are known to be light-induced, in eukaryotic plant cells (see, for example, genetic engineering of plants, an agricultural perspective, a. cashmore, pelham, new york, 1983, pp. 29-38, g. coruzzi et al., j. biol. chem., 258:1399 (1983), and p. dunsmuir, et al., j. molecular and app. gen., 2:285 (1983)).

15 the expression cassette comprising the structural gene for the protein of this invention operably linked to the desired control sequences can be ligated into a suitable cloning vector. in general, plasmid or viral (bacteriophage) vectors containing replication and control sequences derived from  
20 species compatible with the host cell are used. the cloning vector will typically carry a replication origin, as well as specific genes that are capable of providing phenotypic selection markers in transformed host cells. typically, genes conferring resistance to antibiotics or selected herbicides  
25 are used. after the genetic material is introduced into the target cells, successfully transformed cells and/or colonies of cells can be isolated by selection on the basis of these markers.

typically, an intermediate host cell will be used in the  
30 practice of this invention to increase the copy number of the cloning vector. with an increased copy number, the vector containing the gene of interest can be isolated in significant quantities for introduction into the desired plant cells. host cells that can be used in the practice of this invention  
35 include prokaryotes, including bacterial hosts such as e. coli, s. typhimurium, and serratia marcescens. eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention. since these hosts are also microorganisms, it

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will be essential to ensure that plant promoters which do not cause expression of the protein in bacteria are used in the vector.

the isolated cloning vector will then be introduced into  
5 the plant cell using any convenient technique, including electroporation (in protoplasts), retroviruses, bombardment, and microinjection into cells from monocotyledonous or dicotyledonous plants in cell or tissue culture to provide transformed plant cells containing as foreign dna at least one  
10 copy of the dna sequence of the plant expression cassette. preferably, the monocotyledonous species will be selected from maize, sorghum, wheat or rice, and the dicotyledonous species will be selected from soybean, alfalfa, rapeseed, sunflower or tomato. using known techniques, protoplasts can be  
15 regenerated and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for a protein according to this invention. accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign dna at  
20 least one copy of the dna sequence of an expression cassette of this invention.

finally, this invention provides methods of imparting resistance to diseases caused by microorganisms selected from  
fusarium graminearum, fusarium moniliforme, diplodia maydis,  
25 colletotrichum graminicola, verticillium alboatrum, phytophthora megaspermae f.sp. glycinea, macrophomina phaseolina, diaporthe phaseolorum caulivora, sclerotinia sclerotiorum, sclerotinia trifoliorum, aspergillus flavus to plants of a susceptible taxon, comprising the steps of:

- 30 a) culturing cells or tissues from at least one plant from the taxon,  
b) introducing into the cells or tissue culture at least one copy of an expression cassette comprising a structural gene for one or more of the compounds of this invention,  
35 operably linked to plant regulatory sequences which cause the expression of the compound or compounds in the cells, and  
c) regenerating disease-resistant whole plants from the cell or tissue culture. once whole plants have been obtained,

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they can be sexually or clonally reproduced in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

5 alternatively, once a single transformed plant has been obtained by the foregoing recombinant dna method, conventional plant breeding methods can be used to transfer the structural gene for the compound of this invention and associated regulatory sequences via crossing and backcrossing. such  
10 intermediate methods will comprise the further steps of

a) sexually crossing the disease-resistant plant with a plant from the disease-susceptible taxon;

b) recovering reproductive material from the progeny of the cross; and

15 c) growing disease-resistant plants from the reproductive material. where desirable or necessary, the agronomic characteristics of the susceptible taxon can be substantially preserved by expanding this method to include the further steps of repetitively:

20 a) backcrossing the disease-resistant progeny with disease-susceptible plants from the susceptible taxon; and

b) selecting for expression of antimicrobial activity (or an associated marker gene) among the progeny of the backcross, until the desired percentage of the characteristics  
25 of the susceptible taxon are present in the progeny along with the gene imparting antimicrobial activity.

by the term "taxon" herein is meant a unit of botanical classification of genus or lower. it thus includes genus, species, cultivars, varieties, variants, and other minor taxo-  
30 nomic groups which lack a consistent nomenclature.

it will also be appreciated by those of ordinary skill that the plant vectors provided herein can be incorporated into agrobacterium tumefaciens, which can then be used to transfer the vector into susceptible plant cells, primarily  
35 from dicotyledonous species. thus, this invention provides a method for imparting antimicrobial activity and disease resistance in agrobacterium tumefaciens-susceptible dicotyledonous plants in which the expression cassette is

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introduced into the cells by infecting the cells with agrobacterium tumefaciens, a plasmid of which has been modified to include a plant expression cassette of this invention.

5

#### Human and veterinary pharmaceutical use

This invention also provides methods of treating and preventing infection by susceptible organisms in a human or  
10 lower animal host in need of such treatment, which method comprises administration to the human or lower animal host in need of such treatment a therapeutically effective amount of a polypeptide of this invention or a composition containing one or more of the polypeptides. The polypeptides of the present  
15 invention may be administered parenterally, by inhalation spray, rectally or topically in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants and vehicles as desired. The term parenteral as used herein includes subcutaneous, intravenous,  
20 intramuscular, intraarticular and intrathecal injection and infusion techniques. As with other polypeptides, the polypeptides of this invention are not known to be active orally.

Total daily dose of the compounds of this invention  
25 administered to a host in single or divided doses may be in amounts, for example, of from 1 to 2000 mg/kg body weight daily and more usually 50 to 500 mg/kg. Dosage unit compositions may contain such amounts or fractions or submultiples thereof as appropriate to make up the daily dose.  
30 It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of  
35 excretion, drug combination and the severity of the particular disease undergoing therapy.

This invention also provides pharmaceutical compositions in unit dosage form, comprising an effective amount of a

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compound of this invention in combination with a conventional pharmaceutical carrier. As used herein, the term "pharmaceutical carrier" means a solid or liquid filler, diluent or encapsulating material. Some examples of the materials which can serve as pharmaceutical carriers are sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; polyols such as propylene glycol, glycerin, sorbitol, mannitol and polyethylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution, ethyl alcohol and phosphate buffer solutions, as well as other non-toxic compatible substances used in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, and perfuming agents and preservatives can also be present in the compositions, according to the desires of the formulator. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration.

By "therapeutically effective amount" herein is meant an amount of either polypeptide or combination thereof sufficient to provide antimicrobial activity so as to alleviate or prevent infection by susceptible organisms in the human or lower animal being treated at a reasonable benefit/risk ratio attendant with any medical treatment.

### 35      Antifungal Testing

The antifungal activity of compounds synthesized in accord with this invention was measured using art-recognized methods,

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as described in Duvick et al., J. Biol. Chem. 26:18814-18820 (1992) against *Aspergillus flavus*, *S. sclerotiorum*, *Fusarium graminearum* and *F. moniliforme*. Results are shown in Figures 1 through 4.

5        In Figure 1, the curves are labeled according to their SEQUENCE I.D. Nos., with the exception of the curve labeled "10," which reflects the performance of a crude mixture of correctly and incorrectly folded compounds, including some quantity of the compound having SEQUENCE I.D. No. 2. The two  
10       curves labeled "1" and "1a" represent results obtained with natural and synthetic hordothionin, respectively, both having the sequence shown in SEQUENCE I.D. No. 1.

      In Figures 2 through 4, the curves labeled as "1" and "1a" again represent results obtained with natural and synthetic  
15       hordothionin, respectively, both having the sequence shown in SEQUENCE I.D. No. 1. The curves for other derivatives are labeled according to the SEQUENCE I.D. No. of the derivative.



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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Rao, A. Gururaj; Beach, Larry

(ii) TITLE OF INVENTION: HIGH LYSINE DERIVATIVES OF  $\alpha$ -

## 5 HORDOTHIONIN

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Pioneer Hi-Bred International, Inc.

(B) STREET: 700 Capital Square, 400 Locust  
10 Street

(C) CITY: Des Moines

(D) STATE: Iowa

(E) COUNTRY: United States

(F) ZIP: 50309

15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb  
storage

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: MS-DOS, Microsoft Windows

20 (D) SOFTWARE: Microsoft Windows Notepad

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Roth, Michael J.

30 (B) REGISTRATION NUMBER: 29,342

(C) REFERENCE/DOCKET NUMBER: 0233 US

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (515) 245-3594

(B) TELEFAX: (515) 245-3634

35 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

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(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin

5 (iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE :

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Hordeum vulgare*

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Lys Ser Cys Cys Arg Ser Thr Leu Gly Arg

5 10

Asn Cys Tyr Asn Leu Cys Arg Val Arg Gly

15 20

15 Ala Gln Lys Leu Cys Ala Gly Val Cys Arg

25 30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro

35 40

Thr Gly Phe Pro Lys

20 45

[2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 amino acids

(B) TYPE: amino acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

30 Lys Ser Cys Cys Lys Ser Thr Leu Gly Lys

5 10

Asn Cys Tyr Asn Leu Cys Lys Val Lys Gly

15 20

Ala Gln Lys Leu Cys Ala Gly Val Cys Lys

35                      25                      30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro

35 40

- 17 -

Thr Gly Phe Pro Lys

45

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 45 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

- 10 (A) DESCRIPTION: hordothionin derivative

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg

5

10

Asn Cys Tyr Asn Leu Cys Lys Val Lys Gly

15

15

20

Ala Gln Lys Leu Cys Ala Gly Val Cys Lys

25

30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro

35

40

20 Thr Gly Phe Pro Lys

45

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 45 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (A) DESCRIPTION: hordothionin derivative

- 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg

5

10

Asn Lys Tyr Asn Leu Cys Lys Val Lys Gly

15

20

35 Ala Gln Lys Leu Cys Ala Gly Val Thr Lys

25

30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro

35

40

- 18 -

Thr. Gly Phe Pro Lys

45

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 45 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg

5

10

Lys Lys Tyr Asn Leu Cys Lys Val Lys Gly

15

15

20

Ala Lys Lys Leu Cys Ala Gly Val Thr Lys

25

30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro

35

40

20 Lys Gly Phe Pro Lys

45

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 45 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Lys Asp Cys Cys Lys Ser Thr Leu Gly Lys

5

10

Lys Lys Tyr Asn Leu Cys Lys Val Lys Gly

15

20

35 Ala Lys Lys Leu Cys Ala Gly Val Thr Lys

25

30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro

35

40

- 19 -

Lys Gly Phe Pro Lys

45

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 45 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg

5

10

Lys Cys Tyr Asn Leu Cys Lys Val Lys Gly

15

15

20

Ala Gln Lys Leu Cys Ala Gly Val Cys Lys

25

30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro

35

40

20 Lys Gly Phe Pro Lys

45

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 45 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Lys Ser Cys Cys Lys Ser Thr Leu Gly Arg

5

10

Lys Cys Tyr Asn Leu Cys Lys Val Lys Gly

15

20

35 Ala Lys Lys Leu Cys Ala Gly Val Cys Lys

25

30

Cys Lys Leu Thr Ser Ser Gly Lys Cys Pro

35

40

- 20 -

Lys Gly Phe Pro Lys

45

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 45 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- 10 (A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Lys Xaa Cys Cys Xaa Ser Thr Leu Gly Xaa  
                                   5                                  10  
 Xaa Xaa Tyr Asn Leu Cys Xaa Val Xaa Gly  
 15                                  15                                  20  
 Ala Lys Xaa Leu Cys Ala Gly Val Xaa Xaa  
                                   25                                  30  
 Cys Xaa Leu Thr Ser Ser Gly Xaa Cys Pro  
                                   35                                  40  
 20 Thr Gly Phe Pro Xaa  
                                   45

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## WHAT IS CLAIMED IS:

1. A protein having the sequence of SEQUENCE I.D. No. 9 wherein the amino acid residues at one or more of positions 5, 10, 11, 12, 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are the residues at the corresponding positions in SEQUENCE I.D. No. 1, provided that the residue at position 30 is threonine when the residue at position 12 is lysine and cysteine otherwise, and the residue at position 2 is aspartic acid when the residue at position 10 is lysine and serine otherwise.
2. A protein according to Claim 1 wherein one or more of the amino acid residues at positions 5, 11, 12, 17, 19, 22 and 41 are lysine.
3. A protein according to Claim 2 wherein all of the amino acid residues at positions 5, 11, 12, 17, 19, 22 and 41 are lysine.
4. A nucleotide sequence which codes for a protein according to Claim 1.
5. An RNA sequence according to Claim 4.
6. A DNA sequence according to Claim 4.
7. An expression cassette containing the DNA sequence of claim 6 operably linked to plant regulatory sequences which cause the expression of the DNA sequence in plant cells.
8. A bacterial transformation vector comprising an expression cassette according to Claim 7, operably linked to bacterial expression regulatory sequences which cause replication of the expression cassette in bacterial cells.

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9. Bacterial cells containing as a foreign plasmid at least one copy of a bacterial transformation vector according to Claim 8.

5 10. Transformed plant cells containing at least one copy of the expression cassette of Claim 7.

11. Transformed cells according to Claim 10, further characterized in being cells of a monocotyledonous species.

10

12. Transformed cells according to Claim 11, further characterized in being maize, sorghum, wheat or rice cells.

13. Transformed cells according to Claim 10, further  
15 characterized in being cells of a dicotyledonous species.

14. Transformed cells according to Claim 13, further characterized in being soybean, alfalfa, rapeseed, sunflower, tobacco or tomato cells.

20

15. A maize cell or tissue culture comprising cells according to claim 12.

16. A transformed plant comprising transformed cells  
25 according to Claim 10.

17. A method for killing and inhibiting plant pathogenic microorganisms which are susceptible to a-Hordothionin comprising introducing into the environment of the pathogenic  
30 microorganisms an antimicrobial amount of a protein according to Claim 1.

18. A method for killing and inhibiting plant pathogens selected from Fusarium graminearum, Fusarium moniliforme,  
35 Diplodia maydis, Colletotrichum graminicola, Verticillium alboatrum, Phytophthora megaspermae f.sp. glycinea, Macrophomina phaseolina, Diaporthe phaseolorum caulivora, Sclerotinia sclerotiorum, Sclerotinia trifoliorum, and



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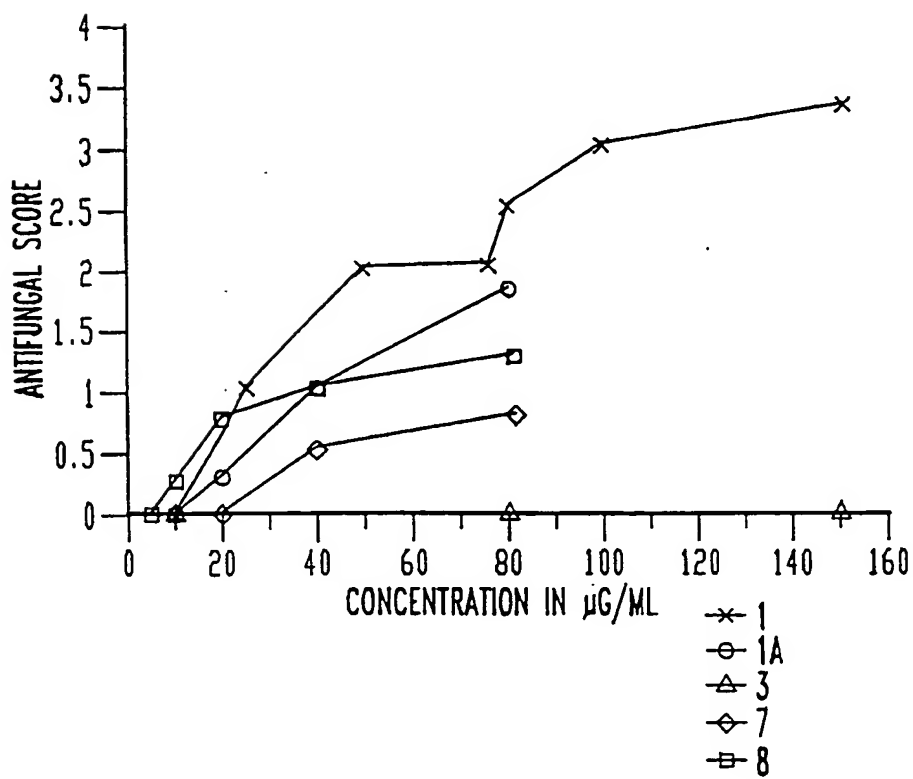
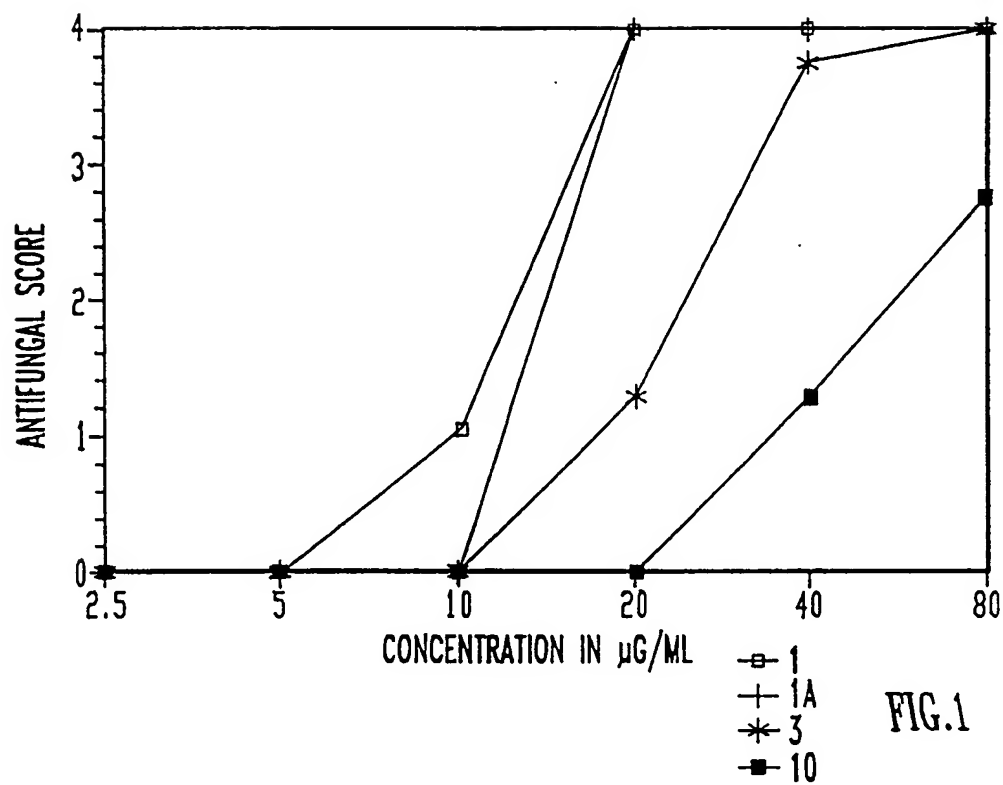
Aspergillus flavus, comprising introducing into the environment of the pathogenic microorganisms an antimicrobial amount of a protein according to Claim 1.

5        19. A method according to Claim 17 wherein the environment of the pathogen is the tissues of a living plant.

20. A method for enhancing the lysine content of a plant cell or seed comprising the step of causing a protein  
10 according to Claim 1 to be expressed in the cell or seed.

21. A method for enhancing the lysine content of a plant comprising the step of causing a protein according to claim 1 to be expressed in tissues of the plant.

1/2



2/2

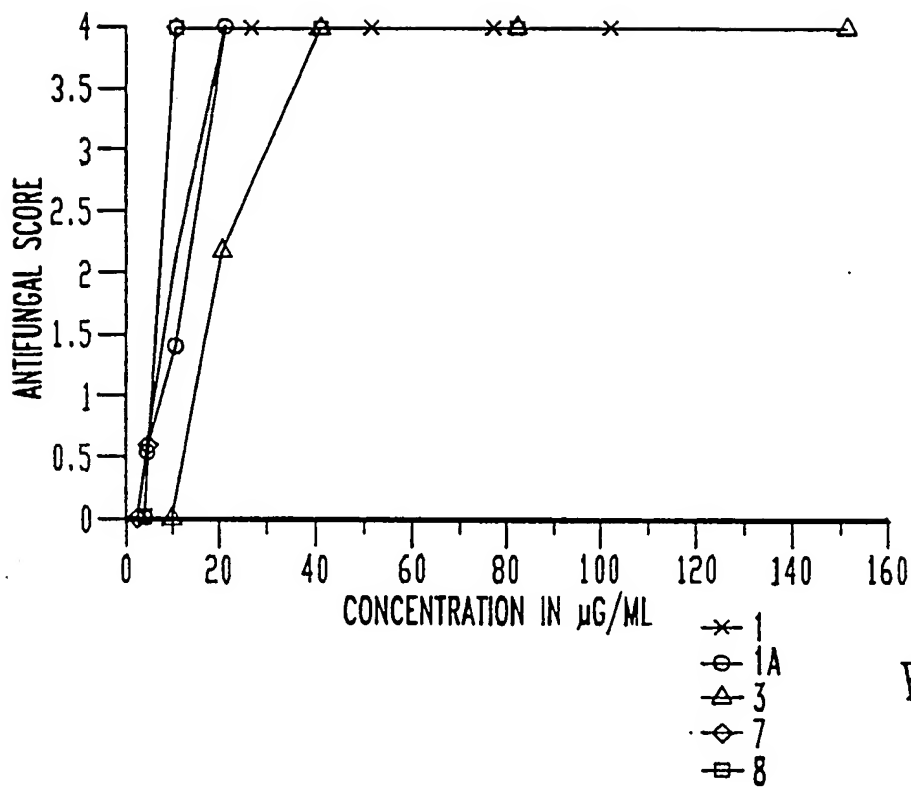


FIG. 3

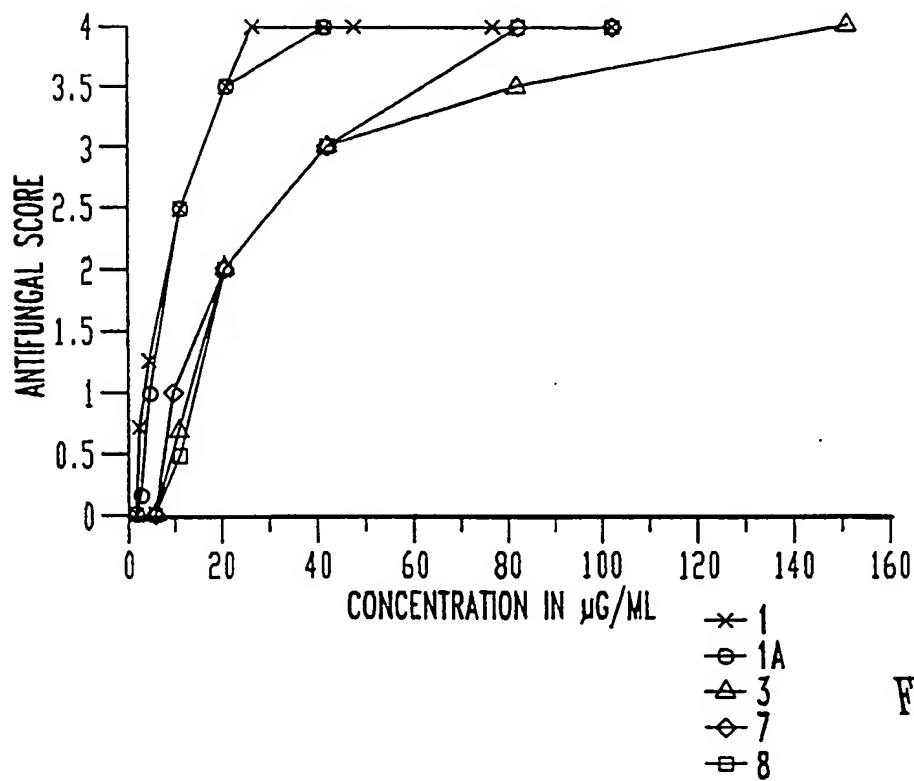


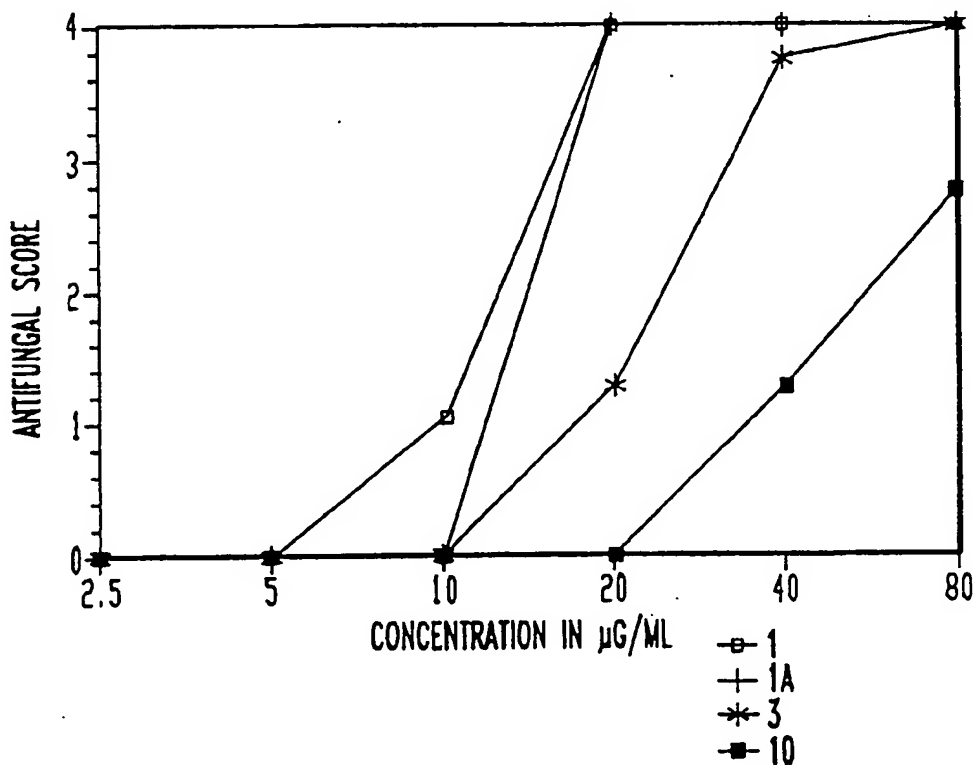
FIG. 4



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : C12N 15/29, C07K 13/00, C12N 5/10, A01H 5/00, A01N 65/00, C12N 1/21		A3	(11) International Publication Number: <b>WO 94/16078</b> (43) International Publication Date: 21 July 1994 (21.07.94)
(21) International Application Number: PCT/US94/00382 (22) International Filing Date: 12 January 1994 (12.01.94) (30) Priority Data: 08/003,885 13 January 1993 (13.01.93) US (71) Applicant: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US). (72) Inventors: RAO, A., Gururaj; 4628 70th Place, Urbandale, IA 50322 (US). BEACH, Larry, R.; 3939 Maquoketa Drive, Des Moines, IA 50311 (US). (74) Agents: ROTH, Michael, J. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 1st September 1994 (01.09.94)	

(54) Title: HIGH LYSINE DERIVATIVES OF ALPHA-HORDOTHIONIN



(57) Abstract

Derivatives of  $\alpha$ -hordothionin made by position-specific substitution with lysine residues provide lysine enrichment while retaining the antifungal activity of the parent compound.

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/29 C07K13/00 C12N5/10 A01H5/00 A01N65/00  
C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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IPC 5 C12N C07K A01N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>PROTEIN ENGINEERING ; SUPPLEMENT, ADVANCES IN GENE TECHNOLOGY: PROTEIN ENGINEERING AND BEYOND. MIAMI WINTER SYMPOSIUM , JAN 17-22, 1993 vol. 6 , 1993 page 117 RAO, A.G., ET AL. 'Validation of the structure-function properties of alpha-hordothionin and derivatives through protein modelling' see abstract</p> <p style="text-align: center;">--- -/--</p>	1-21

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Date of the actual completion of the international search

23 June 1994

Date of mailing of the international search report

12.07.94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CURR. TOP. PLANT PHYSIOL.:BIOSYNTHESIS AND MOLECULAR REGULATION OF AMINO ACIDS IN PLANTS vol. 7 , 1992 pages 229 - 238 BEACH, L.R., ET AL. 'Enhancing the nutritional value of seed crops' see page 233 - page 235 ---</p>	1-21
A	<p>J. CELL. BIOCHEM. SUPPL. vol. 16F , 1992 page 217 MADDOX, J., ET AL. 'Cloning of a barley gene, alpha-hordothionin, and expression in transgenic tobacco' see abstract y212 ---</p>	1-21
A	<p>JOURNAL OF EXPERIMENTAL BOTANY.SUPPLEMENT vol. 42, no. 238 , May 1991 page 4 GARCIA-OLMEDA, F., ET AL. 'Trypsin/alpha-amylase inhibitors and thionins from cereals: possible role in crop protection' see abstract P1.5 ---</p>	1-21
A	<p>EP,A,0 502 718 (PIONEER HI-BRED) 9 September 1992 see the whole document -----</p>	1-21

